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EVALUATION OF IN VITRO ALTERNATIVES TO THE DOG CARDIAC SENSITIZATION ASSAY

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This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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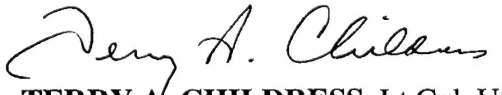

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13. ABSTRACT (Maximum 200 words)

Many halogenated hydrocarbons have been shown to be cardiac sensitizers, i.e., chemicals that alter the sensitivity of the heart to endogenous chemical signals such that under conditions of stress cardiac failure may occur. The best known examples of these agents are solvents and propellants used in aerosol dispensers that have led to the death of "glue sniffers" as a result of cardiac arrest. The standard toxicological test for cardiac sensitization involves the exposure of dogs to the test agent and the recording of the EKG to determine the cardiac response to a systemic injection of epinephrine. In some cases the experimental procedure is lethal. It would be useful, both from a practical as well as a humane point of view, to have non-whole animal test procedures, possibly *in vitro* tests, that would provide quantitative evaluation of the risk for cardiac sensitization of candidate chemicals for Department of Defense applications. This report reviews various *in vitro* models that potentially could be developed for such purposes, including primary cell cultures, cell lines, and isolated organ cultures. At this time, none of the models have been developed for this application. It is apparent that a better understanding of the mechanism of cardiac sensitization would allow for the more rapid development and validation of non-whole animals test systems.

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PREFACE

The literature review described herein began in April 1994, and was completed in July 1994, by Dr. J.M. Frazier, under a subcontract to ManTech Environmental Technology Inc., Toxic Hazards Research Unit (THRU), and was coordinated by Darol E. Dodd, Ph.D., THRU Laboratory Director. This work was sponsored by the Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory, and was performed under Department of the Air Force Contract No. F33615-90-C-0532 (Study No. F33). Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division.

Dr. J.M. Frazier has provided permission to reprint this report herein.

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MODEL SYSTEMS

CARDIAC MYOCYTE CULTURES *IN VITRO*

Beginning in the 1960s myocardial cell cultures were developed for mechanistic studies of cardiac metabolism and function. The following is a summary of some of the mammalian cardiac cell culture systems that have been described in the literature. A significant research effort has been invested in chick embryo myoblast cell culture. However, for the purposes of this project these activities have been excluded because of the problems of extrapolation from avian to mammalian cardiac toxicity that would require extensive validation.

Neonatal Rat Heart Cell Cultures

Wenzel, et al. (1970) investigated the effects of nicotine on cultured neonatal rat heart cells. Using a procedure described by Mark and Strasser (1966) to dissociate heart cells from 2 to 4 day old Sprague-Dawley rats, they evaluated techniques to separate myocardial cells from endothelial cells which tended to overgrow the muscle cell cultures. Having improved the purity of the myocardial cell cultures, they demonstrated that nicotine increased spontaneous cell beating rates. At higher concentrations of nicotine, there was evidence of cytotoxicity (vacuole formation and nuclear swelling).

Schanne, et al. (1977) described the electrogenic properties of a preparation of rat (Sprague-Dawley) neonatal ventricular myocytes derived from 24 to 48 h old rats (the original description of this preparation is in German). Cardiac myocytes derived from the lower third of the neonatal hearts are cultured in streptomycin-free media, since streptomycin was found to interfere with spontaneous electrical activity. Various studies were conducted to characterize the nature of the spontaneous beating activity. The authors concluded that the electrogenic pacemaker activity is a result of a slow, tetrodotoxin insensitive - manganese sensitive, inward current. Some of the properties of this culture system differ from those observed in other model systems.

McCall (1976) describes studies of myocardial cells in culture using the method of Harary and Farley (1963) as modified by Blondel, et al. (1971) to improve the purity of the myocardial cell preparations. The

purpose of the studies was to investigate the effect of quinidine, a drug used to control tachycardia, on cardiac cell function. Using Na flux measurements and cell contraction rates as experimental parameters, McCall was able to demonstrate that quinidine reduced the spontaneous beating rate of the myocardial cells in culture and this effect was correlated with a reduction in Na influx. Na influx and spontaneous beating rate was insensitive to tetrodotoxin (Na channel blocker) and highly sensitive to verapamil (Ca channel - slow inward current blocker). Furthermore, quinidine only affected the verapamil sensitive Na influx.

Welder et al. (1991b) used primary cultures of neonatal myocardial cells to investigate the effects of dietary manipulations during gestation. The investigators used the method of Wenzel et al. (1970) to isolate myocardiac cells. Several observations of this study are important. First, there were significant differences between the cells isolated from neonates of dams given a full-calorie diet during gestation versus those given a 50% calorie-restricted diet. This observation indicates that cells retain treatment-specific characteristics even after isolation. Second, the investigators determined the ß-adrenergic receptor density (receptors per cell) and affinity (K_D) using a radio-ligand binding assay. Since epinephrine acts on cardiac myocytes through the ß-adrenergic receptor, these studies indicate the presence of the appropriate receptor in the cultured cells.

Details of the preparation of neonatal rat myocardial cells is given by Welder and Acosta (1993).

Adult Rat Heart Cell Cultures

Early investigations of adult rat myocardial cell cultures (Vahouny, et al., 1970) indicated that spontaneously beating cells could be obtained and cultured. Heart tissue was minced and treated with digestive enzymes to isolate myocytes. Primary cultures exhibited spontaneous beating rates ranging from 20 to 120 beats per minute at room temperature. The beating rate was highly dependent on medium conditions.

Farmer, et al. (1977) described a different procedure to produce myocyte cultures from adult rats involving the perfusion of multiple rat hearts to increase yield. The cell preparations obtained exhibited a 70% viability. In many respects these cell cultures exhibit normal metabolic regulation but under low Ca culture conditions ([Ca] < 10^{-5} M) the myocytes do not beat spontaneously - random twitching is reported. However, if the calcium concentration is increased, rapid twitching of most cells is observed. This is referred to as the "calcium paradox" and is related to a loss of control of Ca permeability of the cell membrane. The investigators demonstrated that isoproterenol (a $ß_1$-/$ß_2$-adrenergic receptor agonist) increased intracellular cAMP and this effect

was blocked by propranolol (a β_1-adrenergic receptor antagonist) indicating that the β_1-adrenergic receptors, through which epinephrine acts, was present and functional.

Welder, et al. (1991a) recently described an improved perfusion technique to obtain adult rat myocytes. Their procedure is based on previous work by Farmer, et al. (1983) and Khetarpal, et al. (1988). This system was used to investigate the cardiac toxicity of amitriptyline, a tricyclic antidepressant.

Rabbit Heart Cell Cultures

Dani, et al. (1979) developed a perfusion technique to isolate myocardiac cells from the rabbit heart. The cell membrane of the cultured myocytes was electrochemically shunted and freely permeable to Ca, therefore the cells did not exhibit spontaneous beating. The objective of these studies was to investigate calcium transport in relation to contractile activity and this preparation proved useful for this purpose.

Canine Heart Cell Cultures

Vahouny, et al. (1979) described a procedure to isolate canine ventricular myocytes, which is based on their procedure to isolate adult rat heart cells (Vahouny, et al., 1970). Cells isolated by this technique lost their spontaneous beating activity rapidly, within the first hour, but could be stimulated to contract with high Ca concentrations (calcium paradox), ouabain and epinephrine.

Recent Applications

In vitro cellular model systems have been used extensively for basic cardiac research activities. A review of the recent literature (1992-1994) is summarized in Table 1.

Conclusions

There is a significant history of the isolation and culture of myocardial cells from various mammalian species. Over the years the techniques have improved significantly such that current cell culture systems retain the ability to spontaneously beat in the absence of artificial manipulations of culture conditions. It appears that

cultures of either neonatal rat heart myoblasts or adult rat myocytes could form the basis of an experimental *in vitro* test system to investigate the cardiac sensitization potential of chemicals. Any model system selected for further investigation should be characterized with respect to expression and response of $ß_1$-adrenergic receptors (the target of epinephrine) and Na and Ca fluxes before cardiac sensitization validation studies can be initiated.

TABLE 1

Model	Life Stage	Species	Reference
Myocardial cells	Fetal	Human	Goldman and Wurzel, 1992
Cardiomyocytes	Neonatal	Rat	Flanders et al., 1993; Yamazaki et al., 1993 Ito et al. 1993 Suzuki et al., 1993 Hilenski et al., 1992
Ventricular cardiomyocytes	Neonatal	Rat	Orita et al., 1993 Atkins et al., 1992 Dostal et al., 1992 Ogawa et al., 1992
Ventricular cardiomyocytes	Neonatal	Canine	Liu et al., 1992
Cardiomyocytes	Adult	Rat	Clark et al., 1993 Venema and Kuo, 1993 Hartmann and Schrader, 1992 Sadoshima et al., 1992a Sadoshima et al., 1992b Blank et al., 1992 Springhorn et al., 1992 Schulz et al., 1992
Cardiomyocytes	Adult	Rabbit	Hung and Lew, 1993 Liu et al. 1993
Cardiomyocytes	Adult	Canine	Youker et al., 1992
Cardiomyocytes	Adult	Feline	Woosley et al., 1993
Ventricular cardiomyocytes	Adult	Guinea pig	Failli et al., 1992 Walsh and Long, 1992 Adamantidis et al., 1992
Ventricular cardiomyocytes	Adult	Rat	Eid et al., 1992

CARDIAC SUBCELLULAR ORGANELLES

Several studies have utilized subcellular organelles derived from cardiac tissues to investigate mechanisms of cardiac function. Studies on cardiac mitochondrial function are probably not relevant to the issue of cardiac sensitization. However, models for calcium transport processes may be useful if the membrane effects of sensitizing agents alter calcium pump activity and/or regulation. Recent studies have utilized canine sarcolemma vesicles (Qu, et al., 1992) and rat ventricular sarcoplasmic reticulum (Pessah, et al., 1992). These systems may be useful for mechanistic studies.

ISOLATED HEART TISSUES

Heart tissues have been explored as research models for cardiac studies. Minces of heart have been employed in receptor binding studies (Hull, et al., 1993). Many studies, summarized in Table 2, utilize intact tissue, mostly atrial tissue, from adult animals as the research model. These preparations have advantages over isolated cell cultures for electrophysiological studies of signal conduction *in situ*.

ISOLATED HEART PREPARATION

The isolated heart preparation has been used for many years to study regulation of cardiac function. The system has the advantage of maintaining the integrated architecture of the entire heart. Electrophysiological studies can be conducted to measure action potential conduction velocities that could be relevant to the cardiac sensitization question. Some recent studies described in the literature (1992-1994) are listed in Table 3.

SPECIAL PREPARATIONS

Several experimental models have been developed for special research projects to investigate mechanistic questions concerning action potential initiation and conduction. These models are listed in Table 4.

TABLE 2

MODEL	SPECIES	REFERENCES
Isolated atrial strips	Rat	Salvatici et al., 1992a
Spontaneously beating atria	Rat	Schaefer et al., 1992 Agnoletti et al., 1992 Ward et al., 1992 Salvatici et al., 1992b
Isolated atria	Rabbit	Cusack et al., 1993
Spontaneously beating atria	Guinea pig	Lechner,1993 Wilhelm et al., 1992 Eglen et al., 1992

TABLE 3

MODEL	SPECIES	REFERENCES
Isolated perfused heart	Rat	Klabunde et al., 1992 Uusimaa et al., 1992 Van Wylen et al., 1992
Isolated perfused heart	Mouse	Ganim et al., 1992
Isolated perfused heart	Rabbit	Moller and Covino, 1993; Ballagi et al., 1992
Isolated perfused heart	Guinea pig	Gallenberg et al., 1993

TABLE 4

MODEL	SPECIES	REFERENCES
Isolated cardiac ganglia	Rat	Selyanko, 1992
Cardiac Purkinje fibers	Canine	Hanck and Sheets, 1992; Phillips et al., 1992
Cardiac Purkinje fibers	Sheep	Pacini et al., 1992 Rowan et al., 1992 Abete and Vassalle, 1992
Isolated perfused SA node	Guinea pig	Gonzalez and Vassalle, 1993

IN VITRO ASSAYS AS ALTERNATIVES

Cardiac sensitization by halogenated hydrocarbons appears to be a result of a physical-chemical interaction of the toxic agent with the heart tissues. The ultimate effect appears to be an increase in the sensitivity of the cardiac myocytes to the effect of epinephrine resulting in arrhythmia and potentially ventricular fibrillation. The observation of a critical concentration of the chemical in the plasma below which the effect does not occur and the immediate reversibility of the effects when the sensitizing agent is removed argue strongly in favor of a mechanistic hypothesis that the effect is a direct action of the chemical on membrane structure of cardiac cells, although other systemic effects cannot be ruled out completely. The development of an *in vitro* assay as an alternative to the *in vivo* dog assay for the detection of cardiac sensitization requires development and validation of a system that retains the essential cellular components of the process.

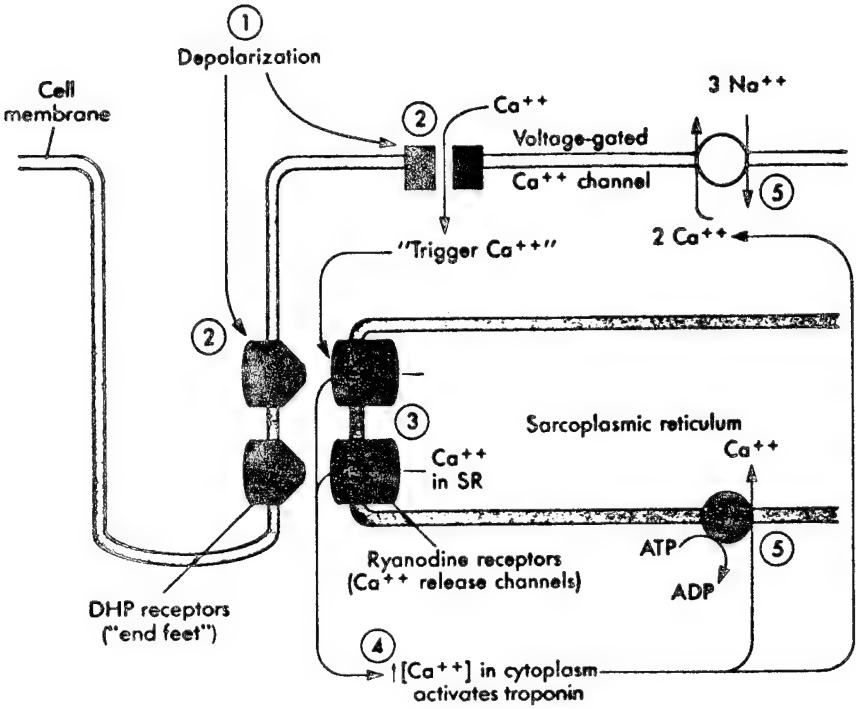
A basic schematic of the processes involved in cardiac muscle contraction are illustrated in Figure 1. The main components of this process are depolarization, regulation of calcium release, activation of the molecular elements of the contraction system (troponin - actin - myosin), and repolarization/restoration of calcium reservoirs. The key characteristic of the cardiac sensitization effect is the increased sensitivity of the heart to epinephrine. The cardiac effects of epinephrine are twofold - an effect on the rate of contraction, and an effect on the strength of contraction. Epinephrine acts on heart tissue through the β_1-adrenergic receptor (Figure 2). The response of cardiac tissues to epinephrine at the cellular level is mediated through cyclic AMP. Thus, the cardiac sensitizing chemical could affect the highly regulated response of cardiac myocytes to epinephrine at any stage of the signal transduction-myocyte contraction continuum. Mechanistic studies should focus on the effects of known cardiac sensitizers on signal transduction through the β_1-adrenergic receptor and the regulation of ion fluxes during spontaneous contraction.

Requirements for an *in vitro* model for the detection of a cardiac sensitizing agent include a cardiac culture system that:

(1) exhibits synchronized spontaneous beating,

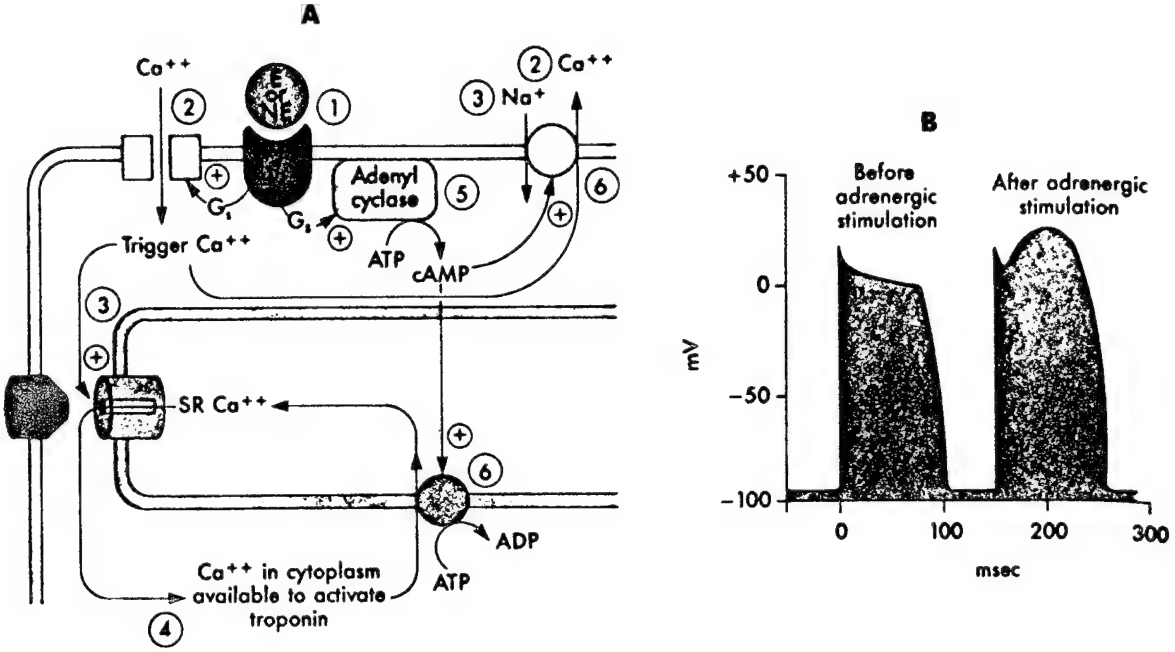
(2) is responsive to epinephrine,

(3) exhibits sensitivity to known cardiac sensitizers, and



Pathways of excitation-contraction coupling in myocardial cells. Depolarization (1) opens (2) both membrane voltage-gated Ca^{++} channels and SR Ca^{++} channels controlled by T tubule DHP receptors. Entry of trigger Ca^{++} potentiates release of (3) Ca^{++} from the SR (Ca^{++}-induced Ca^{++} release). The number of crossbridges that become active is determined by the amount of Ca^{++} released into the cytoplasm (4). Relaxation occurs as Ca^{++} is removed from the cytoplasm (5) by the Ca^{++} pump of the SR and by Na^{+}/Ca^{++} exchange across the cell membrane.

Figure 1: General schematic of processes involved in muscle contraction. Taken from *Human Physiology*, Second Edition, David F. Moffett, Stacie B. Moffitt and Charles L. Schauf, Mosby, St. Louis, 1993.



A Mechanisms of sympathetic inotropic effect. Binding of epinephrine or norepinephrine (1) activates the beta$_1$ receptor, sending a G_s signal to (2) Ca^{++} channels, resulting in an increase in the amount of trigger Ca^{++} that enters during the plateau phase. This induces a greater release of Ca^{++} from the SR when (3) Ca^{++} release channels are activated by depolarization. The ultimate result is that (4) cytoplasmic Ca^{++} rises higher and more cross-bridges are activated, making systole more forceful. The cAMP second message (5) set in motion by the receptor stimulates Ca^{++} pumping (6), making systole briefer.
B Two action potentials from the same myocardial cell before (left) and after (right) adrenergic stimulation. The effect of activating beta$_1$ receptors increases the Ca^{++} current that flows during the plateau, heightening it.

Figure 2: The influence of the ß1-adrenergic receptor on muscle contraction. Taken from *Human Physiology*, Second Edition, David F. Moffett, Stacie B. Moffitt and Charles L. Schauf, Mosby, St. Louis, 1993.

(4) can be cultured in a controlled atmosphere where the system can be exposed to the agents of concern, mostly volatile organics.

The establishment of such a system is technically feasible considering the current state of knowledge in the field.

In the context of the long-term goal to develop new extinguishing agents, the development of a validated *in vitro* screening test system to identify and rank the cardiac sensitizing potential of candidate agents is a high priority. The time and cost ultimately saved by such a test system will far outweigh the initial investment in development and validation. The value of mechanistic studies should not be underestimated. As a result of our lack of understanding of the mechanistic basis of the cardiac sensitization process, the development of an *in vitro* screen is based on intuition and can only be validated empirically by mathematical correlations. This limits the reliability of model predictions and introduces uncertainties that may not be acceptable to regulatory agencies. In this case the currently accepted animal test, the canine cardiac sensitization test, will still be required for meeting regulatory standards. This does not mean that the alternative test cannot be used effectively for screening purposes with the prospect that chemicals with low toxicity *in vitro* will easily pass the *in vivo* test. With a better understanding of the mechanisms involved in the cardiac sensitization process, better *in vitro* tests can be developed that ultimately will gain regulatory acceptance in addition to their value as screening tools.

REFERENCES

Abete, P., Vassalle, M. 1992. Role of intracellular Na+ activity in the negative inotropy of strophanthidin in cardiac Purkinje fibers. *Eur. J. Pharmacol.* **211(3):** 399-409.

Adamantidis, M.M., Adnet-Bonte.C., Adnet, P., Durocher, A., Dupuis, B.A. 1992. Sodium lactate reversal of electrophysiological effects of imipramine in guinea-pig ventricular myocardium. *Fundam. Clin. Pharmacol.* **6(3):** 113-121.

Agnoletti, G., Rodella, A., Cornacchiari, A., Panzali, A.F., Harris, P., Ferrari, R. 1992. Isoproterenol induces release of atrial natriuretic peptide from rat atrium in vitro. *Am. J. Physiol.* **262(1 Pt 2):** H285-292.

Atkins, D.L., Rosenthal, J.K., Krumm, P.A., Marvin, W.J. Jr. 1992. Differential growth of neonatal WKY and SHR ventricular myocytes within sympathetic co-cultures. *J. Mol. Cell. Cardiol.* **24(12):** 1479-1489.

Ballagi-Pordany, G., Nemeth, M., Aranyi, Z., Kekesi, E., Koltai, M.Z., Papp, G., Pogatsa, G. 1992. Effect of glimepiride on the electrical activity of isolated rabbit heart muscle. *Arzneimittelforschung.* **42(2):** 111-113.

Blank, P,S., Silverman, H.S., Chung, O.Y., Hogue, B.A., Stern, M.D., Hansford, R.G., Lakatta, E.G., Capogrossi, M.C. 1992. Cytosolic pH measurements in single cardiac myocytes using carboxy-seminaphthorhodafluor-1. *Am. J. Physiol.* **263(1 Pt 2):** H276-284.

Blondel, B., Roijen, I. and Cheneval, J.P. 1971. Heart cells in culture: A simple method for increasing the proportion of myoblasts. *Experientia* **27**: 356-358.

Clark, W.A., Rudnick, S.J., Simpson, D.G., LaPres, J.J., Decker, R.S. 1993. Cultured adult cardiac myocytes maintain protein synthetic capacity of intact adult hearts. *Am. J. Physiol.* **264(2 Pt 2):** H573-582.

Cusack, B.J., Mushlin, P.S., Voulelis,L.D., Li, X., Boucek, R.J. Jr; Olson, R.D. 1993. Daunorubicin-induced cardiac injury in the rabbit: a role for daunorubicinol? *Toxicol. Appl. Pharmacol.* **118(2):** 177-185.

Dani, A.M., Cittadini, A. and Inesi, G. 1979. Calcium transport and contractile activity in dissociated mammalian heart cells. *Am. J. Physiol.* **6**: C147-C155.

Dostal, D.E., Rothblum, K.N., Conrad, K.M., Cooper, G.R., Baker, K.M. 1992. Detection of angiotensin I and II in cultured rat cardiac myocytes and fibroblasts. *Am. J. Physiol.* **263(4 Pt 1):** C851-863.

Eglen, R.M., Harris, G.C., Cox, H., Sullivan, A.O., Stefanich, E., Whiting, R.L. 1992. Characterization of the interaction of the cervane alkaloid, imperialine, at muscarinic receptors in vitro. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **346(2):** 144-151.

Eid, H., Larson, D.M., Springhorn, J.P., Attawia, M.A., Nayak, R.C., Smith, T.W., Kelly, R.A. 1992. Role of epicardial mesothelial cells in the modification of phenotype and function of adult rat ventricular myocytes in primary coculture. *Circ. Res.* **71(1):** 40-50.

Failli, P., Fazzini, A., Franconi, F., Stendardi, I., Giotti, A. 1992. Taurine antagonizes the increase in intracellular calcium concentration induced by alpha-adrenergic stimulation in freshly isolated guinea-pig cardiomyocytes. *J. Mol. Cell. Cardiol.* **24(11):** 1253-1265.

Farmer, B.B., Harris, T.A., Jolly, W.W., Hathaway, D.R., Katzberg, A., Watanebe, A.M., Whithlow, A.L. and Besch, H.R. Jr. 1977. Isolation and characterization of adult rat heart cells. *Arch. Biochem, Biophysics* **179**: 545-558.

Farmer, B.B., Mancina, M., Williams, E.S., et al. 1983. Isolation of calcium tolerant myocytes from adult rat hearts: Review of the literature and description of a method. *Life Sci.* **33**: 1-18.

Flanders, K.C., Winokur, T.S., Holder, M.G., Sporn, M.B. 1993. Hyperthermia induces expression of transforming growth factor-beta s in rat cardiac cells in vitro and in vivo. *J. Clin. Invest.* **92(1):** 404-410.

Gallenberg, L.A., Stowe, D.F., Kampine, J.P., Bosnjak, Z.J. 1993. Effects of nifedipine with isoflurane, halothane, or enflurane on automaticity, conduction, and contractility in isolated guinea pig hearts. *Anesthesiology* **78(6):** 1112-1119.

Ganim, J.R., Luo, W., Ponniah, S., Grupp, I., Kim, H.W., Ferguson, D.G., Kadambi, V., Neumann, J.C., Doetschman, T., Kranias, E.G. 1992. Mouse phospholamban gene expression during development in vivo and in vitro. *Circ. Res.* **71(5):** 1021-30.

Goldman, B.I., Wurzel, J. 1992. Effects of subcultivation and culture medium on differentiation of human fetal cardiac myocytes. *In Vitro Cell. Dev. Biol.* **28A(2):** 109-119.

Gonzalez, M.D., Vassalle, M. 1993. Role of oscillatory potential and pacemaker shifts in digitalis intoxication of the sinoatrial node. *Circulation* **87(5):** 1705-1714.

Hanck, D.A., Sheets, M.F. 1992. Extracellular divalent and trivalent cation effects on sodium current kinetics in single canine cardiac Purkinje cells. *J. Physiol. Lond.* **454**: 267-298.

Harary, I. and Farley, B. 1963. In vitro studies of single beating rat heart cells. 1. Growth and organization. *Exp. Cell Res.* **29**: 451-465.

Hartmann, M., Schrader, J. 1992. Protein kinase C phosphorylates a 15 kDa protein but not phospholamban in intact rat cardiac myocytes. *Eur. J. Pharmacol.* **226(3):** 225-231.

Hilenski, L.L., Ma, X.H., Vinson, N., Terracio, L., Borg, T.K. 1992. The role of beta 1 integrin in spreading and myofibrillogenesis in neonatal rat cardiomyocytes in vitro. *Cell Motil. Cytoskeleton.* **21(2):** 87-100.

Hull, K.M., Sanderson, W,D., Maher, T,J. 1993. Effect of phenylpropanolamine and related compounds on beta-adrenoceptor-induced activation of adenylyl cyclase. *Life-Sci.* **52(15):** 1287-1294.

Hung, J., Lew, W.Y. 1993. Cellular mechanisms of endotoxin-induced myocardial depression in rabbits. *Circ-Res.* **73(1):** 125-134.

Ito, H., Hiroe, M., Hirata, Y., Tsujino, M., Adachi, S., Shichiri, M., Koike, A., Nogami, A., Marumo, F. 1993. Insulin-like growth factor-I induces hypertrophy with enhanced expression of muscle specific genes in cultured rat cardiomyocytes. *Circulation.* **87(5):** 1715-1721.

Khetarpal, V.K., Kocsis, J.J. and Weiss, A.J. 1988. An improved method for isolating Ca^{2+}-resistant myocytes from the adult rat heart. *Res Commun. Chem. Pathol. Pharmacol.* **60**: 117-120.

Klabunde, R.E., Kimber, N.D., Kuk, J.E., Helgren, M.C., Forstermann, U. 1992. NG-methyl-L-arginine decreases contractility, cGMP and cAMP in isoproterenol-stimulated rat hearts in vitro. *Eur. J. Pharmacol.* **223(1):** 1-7.

Lechner, R.B. 1993. Naloxone potentiates inotropic but not chronotropic effects of isoproterenol in vitro. *Circ. Shock* **39(3):** 226-230.

Liu, K., Massaeli, H., Pierce, G.N. 1993. The action of oxidized low density lipoprotein on calcium transients in isolated rabbit cardiomyocytes. *J. Biol. Chem.* **268(6):** 4145-4151.

Liu, W., Clarkson, C.W., Yamasaki, S., Chang, C., Stolfi, A., Pickoff, A.S. 1992. Characterization of the rate-dependent effects of ethmozine on conduction, in vivo, and on the sodium current, in vitro, in the newborn heart. *J. Pharmacol. Exp. Ther.* **263(2):** 608-616.

Mark, G.E. and Strasser. F.F. 1966. Pacemaker activity and mitosis in culture of newborn rat heart ventricle cells. *Exp. Cell Res.* **44**: 217-233.

McCall, D. 1976. Effect of quinidine and temperature on sodium uptake and contraction frequency of cultured rat myocardial cells. *Cir. Res.* **39**: 730-735.

Moller, R.A., Covino, B.G. 1993. Cardiac electrophysiologic effects of articaine compared with bupivacaine and lidocaine. *Anesth-Analg.* **76(6):** 1266-1273.

Ogawa, S., Barnett, J.V., Sen, L., Galper, J.B., Smith, T.W., Marsh, J.D. 1992. Direct contact between sympathetic neurons and rat cardiac myocytes in vitro increases expression of functional calcium channels. *J. Clin. Invest.* **89(4):** 1085-1093.

Orita, H., Fukasawa, M., Hirooka, S., Fukui, K., Kohi, M., Washio, M. 1993. A cardiac myocyte culture system as an in vitro experimental model for the evaluation of hypothermic preservation. *Surg.Today.* **23(5):** 439-443.

Pacini, D.J., Boachie, Ansah, G., Kane, K.A. 1992. Modification by hypoxia, hyperkalaemia and acidosis of the cardiac electrophysiological effects of a range of antiarrhythmic drugs. *Br. J. Pharmacol.* **107(3):** 665-670.

Pessah, I.N., Schiedt, M.J., Shalaby, M.A., Mack, M., Giri, S.N. 1992. Etiology of sarcoplasmic reticulum calcium release channel lesions in doxorubicin-induced cardiomyopathy. *Toxicology* **72(2):** 189-206.

Phillips, G.B., Morgan, T.K. Jr., Lumma, W.C. Jr., Gomez, R.P., Lind, J.M., Lis, R., Argentieri, T., Sullivan, M.E. 1992. Synthesis, cardiac electrophysiology, and beta-blocking activity of novel arylpiperazines with potential as class II/III antiarrhythmic agents. *J. Med. Chem.* **35(4):** 743-750.

Qu, Y., Torchia, J., Sen, A.K. 1992. Protein kinase C mediated activation and phosphorylation of Ca(2+)-pump in cardiac sarcolemma. *Can. J. Physiol. Pharmacol.* **70(9):** 1230-1235.

Rowan, E.G., Vatanpour, H., Furman, B.L., Harvey, A.L., Tanira, M.O., Gopalakrishnakone, P. 1992. The effects of Indian red scorpion Buthus tamulus venom in vivo and in vitro. *Toxicon.* **30(10):** 1157-1164.

Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T.J., Izumo, S. 1992a. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. *J. Biol. Chem.* **267(15):** 10551-10560.

Sadoshima, J., Takahashi, T., Jahn, L., Izumo, S. 1992b. Roles of mechano-sensitive ion channels, cytoskeleton, and contractile activity in stretch-induced immediate-early gene expression and hypertrophy of cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.* **89(20):** 9905-9909.

Salvatici, R.P., Gallardo, Carpentier, A., Isaacson, R.L., Carpentier, R.G. 1992a. Cardiac inotropic effects of ethanol and calcium-channel modulators. *Alcohol.* **9(4):** 293-297.

Salvatici, R.P., Standen, D.J., Shulz, C.S., Cabezas, M.E., Torres, C.S., Carpentier, R.G. 1992b. Cardiac beta-adrenergic mediated chrono- and inotropic effects of imipramine in vitro. *In. J. Cardiol.* **36(3):** 289-296.

Schaefer, M.E., Allert, J.A., Adams, H.R., Laughlin, M.H. 1992. Adrenergic responsiveness and intrinsic sinoatrial automaticity of exercise-trained rats. *Med. Sci. Sports. Exerc.* **24(8):** 887-894.

Schanne, O.F., Ruiz-Ceretti, E., Rivard, C. and Chartier, D. 1977. Determinants of electrical activity in clusters of cultured cardiac cells from neonatal rats. *J. Mol. Cell. Cardiol.* **9**: 269-283.

Schulz, R., Nava, E., Moncada, S. 1992. Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. *Br. J. Pharmacol.* **105(3):** 575-580.

Selyanko, A.A. 1992. Membrane properties and firing characteristics of rat cardiac neurones in vitro. *J. Auton. Nerv. Syst.* **39(3):** 181-189.

Springhorn, J.P., Ellingsen, O., Berger, H.J., Kelly, R.A., Smith, T.W. 1992. Transcriptional regulation in cardiac muscle. Coordinate expression of Id with a neonatal phenotype during development and following a hypertrophic stimulus in adult rat ventricular myocytes in vitro. *J. Biol. Chem.* **267(20):** 14360-14365.

Suzuki, T., Kumazaki, T., Mitsui, Y. 1993. Endothelin-1 is produced and secreted by neonatal rat cardiac myocytes in vitro. *Biochem. Biophys. Res. Commun.* **191(3):** 823-830.

Uusimaa, P.A., Peuhkurinen, K.J., Hassinen, I.E., Vuolteenaho, O., Ruskoaho, H. 1992. Ischemia stimulates the release of atrial natriuretic peptide from rat cardiac ventricular myocardium in vitro. *Life-Sci.* **50(5):** 365-373.

Vahouny, G.V., Wei, R., Starkweather, R. and Davis, C. 1970. Preparation of beating heart cells from adult rats. *Science* **167**: 1616-1618.

Vahouny, G.V., Wei, R., Tamboli, A. and Albert, W.N. 1979. Adult canine myocytes: Isolation, morphology and biochemical characteristics. *J. Mol. Cell. Cardiol.* **11**: 339-357.

Van Wylen, D.G., Schmit, T.J., Lasley, R.D., Gingell,R.L., Mentzer, R.M. Jr. 1992. Cardiac microdialysis in isolated rat hearts: interstitial purine metabolites during ischemia. *Am. J. Physiol.* **262(6 Pt 2):** H1934-1938.

Venema, R.C., Kuo, J.F. 1993. Protein kinase C-mediated phosphorylation of troponin I and C-protein in isolated myocardial cells is associated with inhibition of myofibrillar actomyosin MgATPase. *J. Biol. Chem.* **268(4):** 2705-2711.

Walsh, K.B., Long, K.J. 1992. Inhibition of heart calcium and chloride currents by sodium iodide. Specific attenuation in cAMP-dependent protein kinase-mediated regulation. *J.Gen.Physiol.* **100(5):** 847-865.

Ward, D.G., Thomas, G.R., Cronin, M.J. 1992. Relaxin increases rat heart rate by a direct action on the cardiac atrium. *Biochem. Biophys. Res. Commun.* **186(2):** 999-1005.

Welder, A.A. and Acosta, D. 1993. Preparation of primary cultures of postnatal rat myocardial cells for toxicological studies. *Meth. Toxicol.* **1A**: 147-158.

Welder, A.A., Grant, R., Bradlaw, J. and Acosta, D. 1991a. A primary culture system of adult rat heart cells for the study of toxicologic agents. *In Vitro Cell. Dev. Biol.* **27A**: 921-926.

Welder, A.A., Grant, R., Kutschke, R.L., Anthony, M., Bradlaw, J. and Acosta, D. 1991b. Effect of maternal calorie-restricted diet on development of the foetal heart, as evaluated in primary cultures of rat myocardial cells. *Fd. Chem. Toxic.* **29**: 445-452.

Wenzel, D.G., Wheatley, J.W. and Byrd, G.D. 1970. Effects of nicotine on cultured rat heart cells. *Toxicol. Appl. Pharmacol.* **17**: 774-785.

Wilhelm, D., Wilffert, B., Janssens, W.J., Leidig, A., Meuter, C., Ebbert, M., Peters, T. 1992. In vitro pharmacology of R 80122, a novel phosphodiesterase inhibitor. *J. Cardiovasc. Pharmacol.* **20(5):** 705-714.

Woosley, R.L., Chen,Y., Freiman, J.P., Gillis, R.A. 1993. Mechanism of the cardiotoxic actions of terfenadine. *JAMA.* **269(12):** 1532-1536.

Yamazaki, T., Tobe, K., Hoh, E., Maemura, K., Kaida, T., Komuro, I., Tamemoto, H., Kadowaki, T., Nagai, R., Yazaki, Y. 1993. Mechanical loading activates mitogen-activated protein kinase and S6 peptide kinase in cultured rat cardiac myocytes. *J. Biol. Chem. 268(16):* 12069-12076

Youker, K., Smith, C.W., Anderson, D.C., Miller, D., Michael, H., Rossen, R.D., Entman, M.L. 1992. Neutrophil adherence to isolated adult cardiac myocytes. Induction by cardiac lymph collected during ischemia and reperfusion. *J.Clin. Invest.*: 89(2): 602-609.